PERCENT MOISTURE AND SEED COAT CHARACTERISTICS OF ALFALFA SEEDS AFTER ARTIFICIAL INOCULATION

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ABSTRACT

Naturally contaminated seeds have low pathogen levels. Most reports on sanitizing efficacy used artificially inoculated seeds. There is no standard procedure for artificially inoculating seeds with bacteria pathogens. This study compared 15 published inoculation procedures as they impact the percent moisture of alfalfa seeds. The percent moisture after drying was similar for the 15 procedures, verifying that the inoculation method had no effect. Using white and ultraviolet (UV) fluorescent light at 360 nm, the physical characteristics of different alfalfa varieties' seed coats were examined. Exposed cotyledon fluoresced under the UV light, making viewing of wrinkled, broken and cracked seed coats easier. The effects of wetting and drying on broken or cracked seed coats were photographed. During inoculation, cracks or breaks in the seed coats became more pronounced and curled away from the cotyledon; thus, bacteria cells in the inocula became trapped in the cracks or under the seed coat. Upon drying, the seed coat did not return to the original position. Condition of the seeds used for artificial inoculation would therefore be expected to impact the result of a decontamination procedure. Examination of seed lots using the UV fluorescent light could potentially be used to remove the cracked and wrinkled seeds.

PRACTICAL APPLICATIONS

Vegetable sprouts can be a vehicle for foodborne illnesses, and seeds are considered the source of contamination. Naturally contaminated seeds have

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low pathogen levels. Reports on the efficacy of sanitizers used artificially inoculated seeds. There is no standard procedure for artificially inoculating seeds, and different inoculation methods were reported. We compared the impact of 15 different procedures on the percent moisture of alfalfa seeds from one lot. The percent moisture after drying was similar for the various procedures. Using white and UV fluorescent light at 360 nm, we examined the physical characteristics of seed coats. Exposed cotyledon fluoresced under the UV light. During inoculation, broken seed coats curled away from the cotyledon, which did not return to the original condition on drying, allowing bacteria to become trapped. The original condition of the seeds used for artificial inoculation would therefore be expected to impact the result of a decontamination procedure.

INTRODUCTION

One means by which raw vegetable sprouts can become contaminated with human bacterial pathogens is by using seeds that carry the bacteria. In order to reduce the number of foodborne outbreaks associated with the consumption of raw sprouts, the U.S. Food and Drug Administration (FDA) (2005) recommends sanitizing seeds before sprouting to remove/reduce the pathogens (National Advisory Committee on Microbiological Criteria for Foods 1999). Montville and Schaffner (2004) compiled the published data on the efficacy of various methods and types of sanitizers studied to reduce/remove the bacteria pathogens from alfalfa seeds. They analyzed which factors influenced the efficacy and the variability of different sanitization protocols and reported that the time the seeds were in contact with the disinfectant and temperature of treatment did not affect the log microbial reduction. However, they did report that the results were more variable for the same chemical treatment when there was more published data by different researchers.

As naturally contaminated seeds typically carry a pathogen level of less than 1 cfu/g (Wu *et al.* 2001; Montville and Schaffner 2004), investigators used artificially inoculated seeds in their challenge studies with a known bacterial count of a pathogen, either *Salmonella*, *Escherichia coli* O157:H7 or *Listeria monocytogenes*, to determine the sanitization efficacy. Montville and Schaffner's 2004 report indicated that there was also variability in the procedure used to inoculate seeds. At the FDA's Sprout Public Meeting (2005), the participants questioned whether the various liquid inoculating protocols (different contact solutions, time of contact and drying time before use) might affect the seeds and the disinfection study results. To date, there are no comparisons of all the different liquid inoculation procedures used for seeds to show that they achieved the same effect. Researchers did not report on the

condition of the seeds before or after inoculation and only examined seeds for placement of the bacteria. In addition, there is no verification that there is consistency in seed lots when researchers obtain the seeds from various sources. The researchers did not report on the visual condition of the seeds both before and after inoculation. There is evidence that during artificial inoculation of seeds with cracked, wrinkled or broken coats, contamination can get between the coat and cotyledon trapping the bacteria and therefore making it difficult for any liquid disinfectant to reach and inactivate the bacterial pathogens (Beuchat 1997; Charkowski *et al.* 2001).

The purposes of this study were to determine if the different inoculation procedures, drying times and storage conditions (without bacterial cells) listed by Montville and Schaffner (2004) affect the percent moisture of the alfalfa seeds and to document that *Salmonella* cells were trapped under damaged alfalfa seed coats during artificial inoculation.

MATERIALS AND METHODS

Inoculation and Drying Protocols of Alfalfa Seeds

The artificial inoculation protocols tested were from the published information cited by Montville and Schaffner (2004). The protocols are summarized in Table 1. The inoculum liquids used in the challenge studies were: (1) 0.1% peptone water (PW) (Jaquette *et al.* 1996; Mazzoni *et al.* 2001; Fett and Cooke 2003; Pierre and Ryser 2006); (2) deionized water with or without added tryptic soy broth (TSB) (Beuchat 1997; Park *et al.* 2000; Beuchat *et al.*, 2001b; Holliday *et al.* 2001; Wu *et al.* 2001); (3) phosphate buffer, pH 7.2

TABLE 1.
SUMMARY OF PROTOCOLS USED TO ARTIFICIALLY INOCULATE ALFALFA SEEDS

1	Inoculum liquid	a. 0.1% peptone b. di-water with or without TSB c. phosphate buffer d. 0.85% NaCl e. TSB or BHIB f. 5% serum albumen
2	Seed : liquid (g/mL)	1:0.5, 1:1, or 1:5 with 1:1 most used
3	Contact time	Ranged from 0.5 to 10 min with 1 min most used
4	Drying protocol	a. on support over wire mesh in bio-hood b. mesh bag in drying dessicator
5	Drying time	Ranged from 4 to 72 h
6	Storage condition	Placed in plastic bags or glass jars kept at either 4C or RT
7	Storage time before use	Used immediately up to >3 months

(Lang *et al.* 2000; Gandhi and Matthews 2003); (4) 0.85% NaCl (Himathongkham *et al.* 2001); (5) growth broth (Palmai and Buchanan 2002; Thayer *et al.* 2003); and (6) 5% horse serum albumen (Holliday *et al.* 2001). The drying protocols and times (Table 1) were followed as described in each published report. All inoculation procedures were done at room temperature.

Alfalfa seeds were obtained from International Specialty Supplies (Cookeville, TN) and were used throughout this study. Samples of 100 g were inoculated using the liquid without bacteria, then dried and the percent moisture was determined.

Percent Moisture Analysis

Percent moisture of the uninoculated, inoculated and dried alfalfa seeds (immediately after inoculation, after drying and at the time of challenge) were performed according to the International Rules for Seed Testing (International Seeds Testing Association, 1990), using a constant high temperature (130 ± 3C) oven (Lab-Line™ Imperial V oven, Alpha Multiservices, Inc., Conroe, TX). The analysis was repeated twice and the results of each determination were averaged ($n = 4$).

Artificial Inoculation of Alfalfa Seeds with a Pathogen for Microscopic Examination

Salmonella enteritidis Anatum F4317, a produce-related isolate, was used for the microscopic artificial inoculation study. An overnight culture was obtained by transferring 0.1 mL of the stock culture to 100 mL of tryptic soy broth (TSB, Becton, Dickenson & Co., Sparks, MD) and incubating on a rotary shaker for 18 h at 37C. A 1:10 dilution in 0.1% PW (Becton, Dickinson & Co.) was made. An aliquot of the 18-h cell suspension was stained with the green fluorescent dye according to the directions from the Live/Dead Viability kit (Molecular Probes, Inc., Eugene, OR). The cell suspension was centrifuged to remove excess dye and the cell pellet resuspended in PW for use in the hydration study.

Microscopic Analysis

Different varieties of alfalfa seeds from the United States Department of Agriculture seed collection were viewed to determine physical characteristics using a Model MZ FLIII stereo fluorescence microscope (Leica Microsystems, Bannockburn, IL) fitted with a model KL1500 (Schott NA, Inc., Auburn, NY) 150 W halogen lamp house equipped with three fiber guides. The ultraviolet (UV) fluorescence at 360 nm and/or green fluorescent protein (GFP) plus fluorescence (GFP2) at 480 nm used excitation from a 50 W Mercury lamp.

Images were collected on a Model DC200 charge coupled device camera (Leica Microsystems, Bannockburn, IL). The width of the view field was 13 mm. Each alfalfa seed variety was placed in a glass Petri dish and was examined for coat characteristics, including damaged coats, using the white, UV and GFP2 light sources. Example digital images were acquired from these samples.

In order to determine the effects of the inoculation procedure, seeds from the lot used for the percent moisture analysis were selected that had cracked or broken coats and were immobilized on conductive carbon adhesive tabs (Electron Microscopy Sciences, Hatfield, PA). The immobilized dry seeds were hydrated for 1 min with the fluorescent stained *Salmonella* suspended in PW, blotted to remove excess liquid and air dried in the biological hood for 48 h. Photographs were taken after each step under the white, UV and GFP lights. This procedure was repeated.

Statistical Analysis

Statistical analysis was performed using ANOVA based on randomized complete block design, and mean separation using the Bonferroni LSD technique (SAS/STAT 9.1, SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

At the 2005 FDA Sprout Safety Public Meeting (2005), discussion occurred regarding the variability of the sanitizer treatment (Montville and Schaffner 2004) and it was suggested that all artificial inoculation procedures be compared using the same seed lot. The percent moisture procedure as followed by seed testing laboratories was used to determine if the inoculated seeds were dried to a consistent moisture level. Presented in Table 2 are the results of the percent moisture analysis of the alfalfa seeds determined after three steps of the protocol (immediately after inoculation; after drying and at time of challenge). Within the contact time of 0.5 to 1 min, the percent moisture increased from 6.6 ± 0.4% to an average ($n = 30$) of 14.4 ± 1.5%, and did not increase any further for the longer inoculation times (5 or 10 min). The average moisture after soaking in the inoculation liquid was 7.8 ± 0.5%. The drying times at room temperature ranged from 4 to 72 h and the percent moisture analysis was done immediately after drying (no storage). Since there was such a wide range in drying times, the results of the moisture analysis preformed after drying were not averaged and they ranged from 5.1 ± 0.9% to 8.7 ± 2.6% (Table 2). Statistical analysis of the moisture levels immediately after drying did show that the results from two stated procedures were statistically different at $P < 0.05$, which were the 5.1 ± 0.9% (lowest) and

TABLE 2.
PERCENT MOISTURE OF ALFALFA SEED BEFORE, IMMEDIATELY AFTER INOCULATION, AFTER SPECIFIC DRYING TIMES AND LENGTH OF STORAGE

Inoculum	Ratio (g : mL)	Contact time (min)	After inoculation	After drying	At time of use (d storage)
0.1% peptone	1:0.5	1 or 10	13.7 ± 0.54	8.2 ± 0.6 after 48 h	8.1 ± 0.4 (5)
				8.2 ± 0.3 after 4 h	8.1 ± 0.4 (10)
				8.7 ± 2.6 after 24 h†	7.5 ± 0.4 (12)
				7.8 ± 0.3 after 48 h	7.6 ± 0.4 (17)
Deionized water	1:1	1	14.1 ± 0.71	7.7 ± 1.1 after 48 h	7.2 ± 0.4 (8)
Deionized water + TSB	1:1	0.5–1	14.8 ± 3.3	7.8 ± 0.4 after 24 h	7.8 ± 0.4 (1)
				7.9 ± 0.9 after 48 h	7.7 ± 0.3 (16)
Phosphate buffer	1:1	1 or 5	14.6 ± 0.62	7.7 ± 0.7 after 18 h	7.7 ± 0.7 (6)
				7.1 ± 0.6 after 24 h	7.4 ± 0.2 (12)
BHIB	1:0.5	1	14.2 ± 0.6	7.4 ± 0.1 after 24 h	7.2 ± 0.4 (15)
TSB	1:1	1	13.9 ± 1.9	5.1 ± 0.9 after 72 h†	5.5 ± 0.6 (18)
0.85% NaCl	1:0.5	1	13.9 ± 0.5	7.3 ± 0.7 after 24 h	7.3 ± 0.6 (7)
5% horse serum albumun	1:1	1	13.4 ± 0.8	7.1 ± 0.45 after 48 h	7.0 ± 0.2 (8)

Average % moisture of all controls 6.6 ± 0.4 before inoculation.
† Statistically different ($P < 0.05$) from the control.

8.7 ± 2.6% (highest) from the uninoculated control (6.6 ± 0.4%). What is more important is the moisture level at the actual time/condition when the researchers performed their studies. The reported time/storage conditions varied and ranged from immediate use after drying (no storage) up to 7 days of storage before use. The moisture levels at the reported time of challenge ranged from 5.5 ± 0.5% to 8.1 ± 0.4% (Table 2). The individual moisture level was compared with the control (6.6 ± 0.4%) and overall the percent moistures of the dried seeds from the different inoculation procedures were not significantly ($P < 0.05$) different. The moisture content range at the time a challenge would be conducted as determined in this study is in agreement with the moisture results (ca. 6.8% moisture) published by Park *et al.* (2000). We identified and compared eight different inoculation liquids and found no differences. When comparing high (5% horse serum albumen) and low (deionized water) organic load inoculation liquids, Holliday *et al.* (2001) also concluded that the type of inoculation liquid and the organic load did not affect the results of the challenge studies. Based on these results, we concluded that the contact time, inoculation liquid (without cells) and drying procedure do not appear to affect the percent moisture of the artificially inoculated seeds.

Alfalfa seed varieties from different sources were examined microscopically for physical characteristics under white light or by UV fluorescence at 360 nm. Using the white light to illuminate the seeds, it was observed that there were broken and/or cracked seed coats in all samples ($n = 17$) examined.

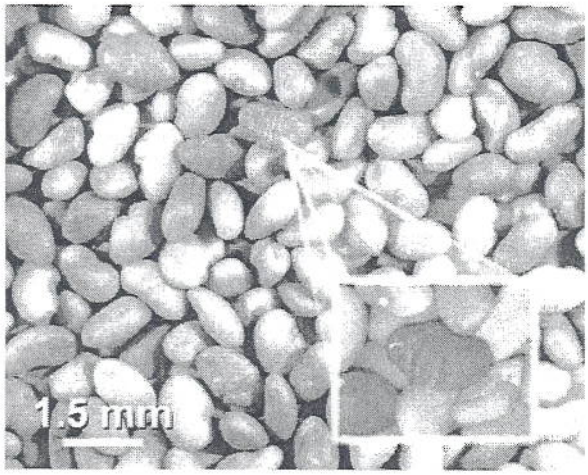

FIG. 1. PHOTOMICROGRAPH OF A REPRESENTATIVE ALFALFA SEEDS AS VIEWED USING WHITE LIGHT AND UV LIGHT AT 360 NM IN INSERT BOX
Insert box (magnification 2x) shows wrinkled seed coat.

The seeds exhibited heterogeneity in size, shape and color, which ranged from light beige to dark brown, with some seeds having a greenish tint, which is typical for alfalfa seeds. A representation is presented in Fig. 1. This heterogeneity of alfalfa seeds used for sprouting was observed by Charkowski *et al.* (2001), who also reported that there was a range in color and size.

When the light source was changed to UV at 360 nm, the exposed cotyledon fluoresced, making cracks, breaks, wrinkles and the hilum (area where seed was attached in the pod) easily identifiable (Fig. 1, insert). Under the white light the wrinkled seed looked intact (Fig. 1), but when viewed under the UV light it was noted that the seed coat was actually cracked (Fig. 1, insert).

Alfalfa seeds with cracked or broken coats were photographed after each liquid inoculation step. The observations were similar for the repeats. In the area where part of the seed coat had broken off, the remaining seed coat appeared to be lifted off the cotyledon (Fig. 2A), the separation becoming more pronounced during hydration. Wade *et al.* (2003) also showed that the broken seed coat was raised and exposed the cotyledon. We observed that during hydration, the coat expanded and exposed more of the cotyledon where there was a break in the seed coat and did not return to the original position upon drying (Fig. 2B). During this time, bacteria in the liquid could enter this exposed area between the seed coat and cotyledon, and then became trapped when dried. Upon blotting after the 1-min contact time with the liquid inoculum, the seed coat curled inward as it started to return to its original position. However, after 48 h of air drying (the drying procedure most used and

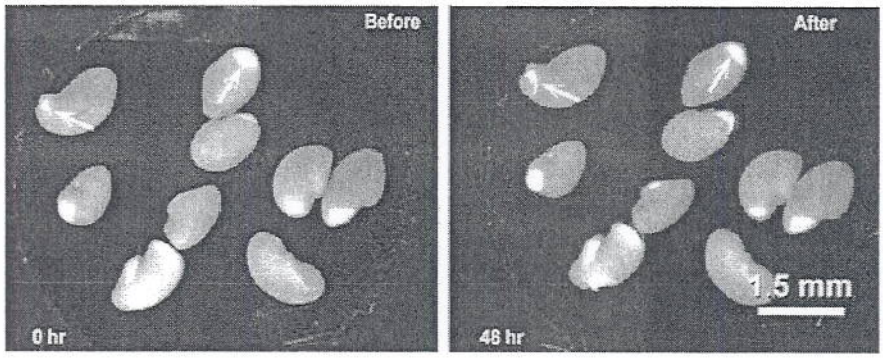

FIG. 2. PHOTOMICROGRAPHS OF ALFALFA SEEDS BEFORE AND AFTER THE 48-H DRYING PROCESS
Before – dry seeds. After air drying for 48 h.

reported), the cracked/broken, curled seed coat had not returned to the original position (Fig. 2b). In some cases, the cotyledon actually expanded and remained exposed after drying (Fig. 2b). The seed, located by the marker bar, is the control intact seed, which exhibited no change in the seed coat during the inoculation procedure.

Using a fluorescent stained *Salmonella* inoculum, the *Salmonella* was observed in the area of the exposed underside of the seed coat, indicated by the arrows in Fig. 3. After this photograph was taken (1 min of contact time), the inoculum was removed by blotting. It was observed that the seed coat was already beginning to close on the cotyledon, entrapping the cells (Fig. 3). After 48 h drying, the seed coat returned to the approximate position before hydration (Fig. 3). Using the stained *Salmonella*, it was observed that the cells did in fact attach to the underside of the exposed seed coat and were trapped under the dried coat.

Since no challenge disinfectant study was successful in totally eliminating the inoculated bacteria from the seed, Beuchat (1997) suggested that the reason sanitizers are ineffective is the inaccessibility to the cells by the disinfectant. Observations during any liquid inoculation procedure of cracked/broken alfalfa seed coats permitting the cells to be trapped, these inoculated bacteria would also be inaccessible. When inoculated alfalfa seeds were viewed using a scanning electron microscope, the bacteria cells were located in the natural crevices of the seed coats, making the cells inaccessible to the sanitizer (Thayer *et al.* 2003).

Based on the observed hydration effect on cracked/broken alfalfa seed coats, the quality of the seeds used in any challenge studies becomes a concern. In the studies reported in Montville and Schaffner's (2004)

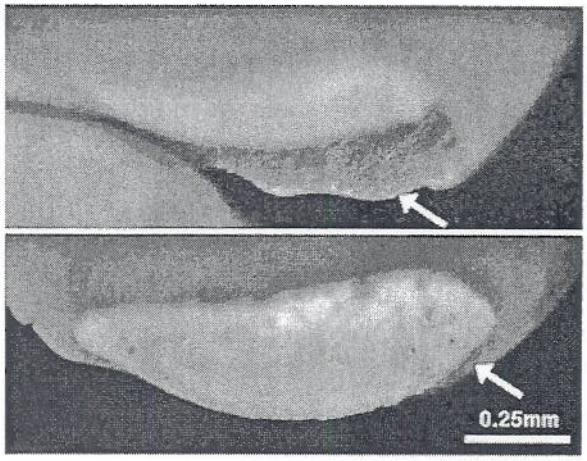

FIG. 3. PHOTOMICROGRAPH OF THE ALFALFA SEED DURING THE INOCULATION WITH FLUORESCENT STAINED *SALMONELLA*
Top – exposed underside of seed coat showing fluorescent *Salmonella,* which are seen as the white dots. Bottom – same seeds showing seed coat closed on the cotyledon entrapping the bacteria and the attached fluorescent *Salmonella* at the edge of seed coat.

comparison, the seeds used were obtained from various sources: two major U.S. seed suppliers, local sprout growers, a local grocery store and unspecified suppliers. Future challenge studies should use good quality seeds with a minimum of cracks/breaks or wrinkles based on the results (Fig. 2) showing that during inoculation, the seed coat separates sufficiently for liquids to become trapped underneath and after drying the seed coat does not return to the original condition. Charkowski *et al.* (2001) reported that the wrinkled alfalfa seeds had higher levels of naturally occurring aerobic bacteria, and that after being artificially inoculated, the seeds had a higher level of contamination than smoother seeds. The results of their challenge studies showed that the wrinkled seeds were more difficult to decontaminate (Charkowski *et al.* 2001), confirming Beuchat's 1997 suggestion that the bacteria were inaccessible to the disinfectant. Holliday *et al.* (2001) also reported that it was more difficult to remove the *Salmonella* from scarified seeds (mechanically cracking the seed coat) than the nonscarified or polished (removal of seed coat layers) alfalfa seeds. They reported mixed results from their challenge studies, and concluded that more testing was needed to determine if indeed scarification impacted the inoculation procedure and efficacy of chemical treatments (Holliday *et al.* 2001).

This study identified 15 different protocols used to artificially inoculate the seeds and five different seed sources. Thayer *et al.* (2003) compared the

physical characteristics (average seed weight and water activity) of alfalfa seeds obtained from two different sources and found that there was a significant difference between the two lots. For future sanitizer challenge studies of artificially inoculated seeds to be compared and conducted, standardization of the seed supply to assure high quality seeds is needed. Such standardization for challenge studies of fresh produce has been proposed and evaluated for inoculation methods and drying times (Lang *et al.* 2004) and for assessing the efficacy of a sanitizer to decontaminate the produce (Beuchat *et al.* 2001a). Development of standard methods to evaluate the efficacy of seed sanitation would enable better comparison of challenge study results. It was suggested that seeds be visually inspected to remove seed lots with a high amount of wrinkled or cracked coats (Charkowski *et al.* 2001). Using an ultraviolet light source at 360 nm, as determined in this study (Fig. 1), such seeds were easily identified. Identification of broken, cracked or wrinkled seeds prior to use in a challenge study would enable researchers to select those lots with a minimum of damaged seeds. Further development studies are needed to determine if the installation of this type of light source in an automatic sorting device would enable the removal of damaged seeds from lots with an appreciable amount of nonintact seed coats. The ability to remove highly damaged seeds from being used for sprouting would be one step in protecting the public from pathogen contaminated alfalfa sprouts.

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